

# Localization of ATP Sulfurylase and O-Acetylserine(thiol)lyase in Spinach Leaves

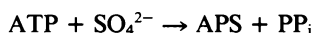
John E. Lunn\*, Michel Droux, Jacqueline Martin, and Roland Douce

Laboratoire mixte CNRS/Rhône-Poulenc, Rhône-Poulenc Agrochimie, 14-20 rue Pierre Baizet, 69009 Lyon, France

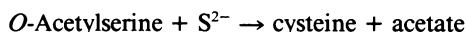
## ABSTRACT

The intracellular compartmentation of ATP sulfurylase and O-acetylserine(thiol)lyase in spinach (*Spinacia oleracea* L.) leaves has been investigated by isolation of organelles and fractionation of protoplasts. ATP sulfurylase is located predominantly in the chloroplasts, but is also present in the cytosol. No evidence was found for ATP sulfurylase activity in the mitochondria. Two forms of ATP sulfurylase were separated by anion-exchange chromatography. The more abundant form is present in the chloroplasts, the second is cytosolic. O-Acetylserine(thiol)lyase activity is located primarily in the chloroplasts and cytosol, but is also present in the mitochondria. Three forms of O-acetylserine(thiol)lyase were separated by anion-exchange chromatography, and each was found to be specific to one intracellular compartment. The cytosolic ATP sulfurylase may not be active *in vivo* due to the unfavorable equilibrium constant of the reaction, and the presence of micromolar concentrations of inorganic pyrophosphate in the cytosol, therefore its role remains unknown. It is suggested that the plant cell may be unable to transport cysteine between the different compartments, so that the cysteine required for protein synthesis must be synthesized *in situ*, hence the presence of O-acetylserine(thiol)lyase in the three compartments where proteins are synthesized.

Higher plants use inorganic sulfate as their major source of sulfur for the synthesis of sulfur containing amino acids, lipids, and coenzymes. The first step in the pathway of sulfate assimilation, (1, 26) involves activation of sulfate by ATP to form APS<sup>1</sup>, catalyzed by ATP sulfurylase (sulfate adenylyltransferase, EC 2.7.7.4):

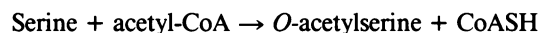


Two possible routes exist for the reduction of the sulfate group of APS. The sulfate group may be transferred to a carrier molecule by APS-sulfotransferase and then reduced to bound sulfide by thiosulfonate reductase. Alternatively the sulfate group may be released as free sulfite and reduced to free sulfide by sulfite reductase (EC 1.8.7.1). Both pathways use reduced ferredoxin as the electron donor. In plants, free or bound sulfide is incorporated into cysteine by O-acetylserine(thiol)lyase (EC 4.2.99.8):



<sup>1</sup> Abbreviations: APS, adenosine 5'-phosphosulfate; NADP-GAPDH, NADP<sup>+</sup> glyceraldehyde-3-phosphate dehydrogenase; PFP, pyrophosphate:fructose-6-phosphate-1-phosphotransferase.

O-Acetylserine is synthesized by the enzyme serine acetyltransferase (EC 2.3.1.30):



In leaves it is known that all the enzymes of the pathway are present in the chloroplasts (1, 25) and that isolated chloroplasts can synthesize [<sup>35</sup>S]cysteine from <sup>35</sup>SO<sub>4</sub><sup>2-</sup> (26). However, O-acetylserine(thiol)lyase is also present outside the chloroplasts (4, 25) and in pea (*Pisum sativum*) roots most of the enzyme is outside the proplastids (8), although the exact location of the extra-plastidial enzyme is unknown. Serine acetyltransferase activity was found in mitochondria from leaves of *Phaseolus vulgaris* (27) in addition to spinach leaf chloroplasts (7).

Compartmentation plays an important role in many plant metabolic pathways (3), but its role in sulfate assimilation is unclear, because the extent of the compartmentation is not known. Therefore, we decided to reexamine the intracellular location of O-acetylserine(thiol)lyase in spinach (*Spinacia oleracea* L.) and pea leaves and investigate whether ATP sulfurylase, the first enzyme in the pathway, is also present in both the chloroplast and extrachloroplastic compartments. We have measured the activities of these enzymes in organelles isolated from leaves, and in fractions obtained by differential centrifugation of protoplast lysates, along with marker enzymes to check for cross-contamination. We have also used anion-exchange chromatography to show the presence of compartment specific forms of ATP-sulfurylase and O-acetylserine(thiol)lyase.

## MATERIALS AND METHODS

### Reagents

Enzymes, ATP, DTT, oxaloacetic acid, acetyl-CoA and nicotinamide cofactors were obtained from Boehringer Mannheim France. All other biochemicals were obtained from Sigma Chimie SARL, La Verpillière, France. Other chemicals were analytical grade. All solutions were made with Milli-Q water.

### Plant Material

Spinach (*Spinacia oleracea* L.) was grown in soil at 18°C with a 12-h photoperiod. Fully expanded leaves from 2-month-old plants were harvested 1 h into the light period. Peas (*Pisum sativum* L.) were grown in vermiculite at 15°C with a 12 h photoperiod. Leaves were harvested from 8 to 15 d-old plants 1 h into the light period.

### Preparation of Spinach Leaf Crude Extract

Spinach leaves (2 g) were homogenized using a mortar and pestle with 0.5 g insoluble PVP and 0.2 g ground quartz in 10 mL 50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 2 mM DTT, 1 mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mM PMSF (pH 7.5). The homogenate was centrifuged at 48,000g for 30 min. The supernatant comprised the crude extract. All procedures were carried out at 4°C.

### Preparation of Chloroplasts

Chloroplasts were prepared from spinach and pea leaves as described by Walker *et al.* (29). Approximately 100 g freshly harvested spinach leaves were homogenized in 800 mL 330 mM sorbitol, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>-HCl (pH 6.5), 5 mM MgCl<sub>2</sub>, 2 mM sodium ascorbate, 0.5% (w/v) BSA, using a Waring blender (3 × 2 s bursts). The brei was squeezed through eight layers of muslin and then passed through a 20 µm nylon mesh. The filtrate was centrifuged at 1,700g for 1 min. The supernatants were discarded and the pellets were resuspended in 10 mL 330 mM sorbitol, 50 mM Hepes-KOH, 2 mM EDTA, 1 mM MgCl<sub>2</sub> (pH 7.6). The chloroplasts were purified by layering 4 mL of the chloroplast suspension onto 4 mL 330 mM sorbitol, 50 mM Hepes-KOH, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 40% (v/v) Percoll (Pharmacia-LKB, Uppsala, Sweden) (pH 7.6), and centrifugation at 1,700g for 1 min (swinging bucket rotor, no brake). The supernatants were discarded and the pelleted intact chloroplasts were lysed by adding 1 mL 50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 2 mM DTT, 1 mM EDTA (pH 7.5) to each pellet. After vortex mixing the chloroplasts were left on ice for 10 min to ensure complete lysis. The lysed chloroplasts were centrifuged at 48,000g for 15 min to remove thylakoid and envelope membranes. The supernatant comprised the chloroplast extract. All procedures were carried out at 4°C.

### Preparation of Mitochondria

Mitochondria were prepared from spinach leaves as described by Edwards and Gardeström (12). Approximately 300 g freshly harvested spinach leaves were homogenized in 1 L 330 mM sucrose, 4 mM DTT, 1 mM EDTA, 30 mM Hepes-NaOH, 0.2% (w/v) insoluble PVP (pH 7.5), using a Waring blender (3 × 2 s bursts). The brei was filtered through a 20 µm nylon mesh, and the filtrate was centrifuged at 5,000g for 2 min. The supernatant was decanted and centrifuged at 20,000g for 2.5 min. The pelleted mitochondria were resuspended in approximately 100 mL 300 mM sucrose, 1 mM EDTA, 10 mM Hepes-NaOH, 0.1% (w/v) BSA (pH 7.5), and centrifuged at 2,000g for 1 min. The supernatant was decanted and centrifuged at 20,000g for 2.5 min. The pelleted washed mitochondria were resuspended in a total volume of 8 mL 330 mM sucrose, 1 mM EDTA, 10 mM Hepes-NaOH, 0.1% (w/v) BSA (pH 7.5). The mitochondria were purified as described by Moore and Proudlove (19) by centrifugation on four linear 0 to 10% (w/v) PVP-25 gradients containing 250 mM sucrose, 10 mM KH<sub>2</sub>PO<sub>4</sub>-KOH, 0.2% (w/v) BSA, 28% Percoll (pH 7.5), in a volume of 40 mL. Two milliliters of the washed mitochondrial suspension were layered onto each gradient, and the gradients were centrifuged at 41,000g for 30 min. The upper part of each gradient containing thylakoids

was removed and the loosely pelleted mitochondria were washed by resuspension in 40 mL 330 mM sucrose, 1 mM EDTA, 10 mM Hepes-NaOH, 0.1% (w/v) BSA (pH 7.5), and centrifugation at 11,000g for 15 min. The supernatants were discarded and the mitochondria were resuspended in 50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 2 mM DTT, 1 mM EDTA, 0.2% (v/v) Triton X-100 (pH 7.5) to give a final volume of 3 mL. After vortex mixing the mitochondria were left on ice for 10 min to ensure complete lysis and then centrifuged at 48,000g for 15 min. The supernatant comprised the mitochondrial extract. All procedures were carried out at 4°C.

Pea leaf mitochondria were prepared as described by Douce *et al.* (11).

### Preparation and Fractionation of Spinach Leaf Protoplasts

Protoplasts were prepared from spinach leaves as described by Robinson (23). Freshly harvested spinach leaves (5 g) were cut into fine strips (0.5–1.0 mm) under incubation medium: 20 mM Mes-NaOH, 0.5 M sorbitol, 1 mM CaCl<sub>2</sub>, 0.05% (w/v) fatty acid free BSA (pH 5.5). The medium was removed and replaced with 100 mL fresh medium containing 2% (w/v) cellulase Onozuka R 10 and 0.3% (w/v) Macerozyme R 10 (Yakult Honsha Co, Shingikancho, Nishinomiya, Japan). After vacuum infiltration the leaf strips were incubated at 25°C in the light for 2.5 h. All subsequent procedures were carried out at 4°C. Protoplasts were released from the digested tissue by gentle shaking, and then separated from the leaf debris by filtering through a 100 µm nylon mesh (Zürcher Beuteltuchfabrik AG, Rüschlikon, Switzerland). The filtrate was centrifuged at 100g for 5 min (swinging bucket rotor) and the supernatant was discarded. The pelleted protoplasts were resuspended in a total volume of 20 mL 0.5 M sucrose, 1 mM CaCl<sub>2</sub>, 5 mM Mes-NaOH (pH 6.0) and divided among four 15-mL glass centrifuge tubes. To each tube was added 2 mL 0.4 M sucrose, 0.1 M sorbitol, 1 mM CaCl<sub>2</sub>, 5 mM Mes-NaOH (pH 6.0) and 1 mL 0.5 M sorbitol, 1 mM CaCl<sub>2</sub>, 5 mM Mes-NaOH (pH 6.0). The resulting three-step gradients were centrifuged at 250g (swinging bucket rotor, no brake) for 5 min. The protoplasts collected at the interface between the top two layers were removed using a Pasteur pipette, suspended in 40 mL 0.5 M sorbitol, 1 mM CaCl<sub>2</sub>, 5 mM Mes-NaOH (pH 6.0) and centrifuged at 100g (swinging bucket rotor) for 5 min. The supernatants were discarded and the purified protoplasts were resuspended in 50 mM Hepes-NaOH, 0.5 M sorbitol, 0.1% BSA (pH 7.5), at a Chl concentration of 0.2 mg mL<sup>-1</sup>.

Protoplasts were ruptured by passing twice through a 20 µm nylon mesh attached to a 1-mL syringe. Chloroplasts were pelleted by centrifuging aliquots (1 mL) of the lysed protoplast suspension at 100g for 5 min. The supernatant was decanted and centrifuged at 5000g for 5 min to give the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant). The chloroplast and mitochondrial pellets were resuspended in 1 mL 50 mM Hepes-NaOH, 0.5 M sorbitol, 0.1% (w/v) BSA (pH 7.5).

For chromatography on Mono Q, 3 mL lysed protoplasts were centrifuged at 5000g for 5 min to give chloroplast + mitochondrial (pellet) and cytosolic (supernatant) fractions. The pelleted chloroplasts and mitochondria were lysed by

resuspension in 3 mL 10 mM Hepes-NaOH, 5 mM MgCl<sub>2</sub> (pH 7.5) and incubation on ice for 5 min, then centrifuged at 48,000g for 10 min. The supernatant comprised the chloroplast + mitochondrial fraction. A total protoplast extract was prepared by addition of Triton X-100 to 3 mL protoplasts to give a final concentration of 0.2% (v/v). After incubation on ice for 5 min the lysed protoplasts were centrifuged at 48,000g for 10 min. The supernatant comprised the total protoplast extract.

#### Fractionation of Spinach Leaf Extracts by Anion Exchange Chromatography

Samples were desalted using a PD 10 Sephadex G-25 (Pharmacia) column equilibrated with 50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 2 mM DTT, 1 mM EDTA (pH 7.5), and filtered (0.2  $\mu$ m cellulose acetate) before loading onto the Q Sepharose or Mono Q HR 5/5 columns, that had been equilibrated with the same buffer.

#### Q Sepharose Chromatography

Samples (2 mL) of spinach leaf chloroplast, mitochondrial and crude extracts were fractionated on a Q Sepharose Fast-Flow (Pharmacia) column (50  $\times$  16 mm diameter) attached to a fast protein liquid chromatography (FPLC) system. After sample loading the column was washed with 10 mL equilibration buffer and then eluted with a gradient of 0 to 0.5 M NaCl (100 mL), 0.5 to 1.0 M NaCl (50 mL) in the same buffer, at a flow rate of 0.2 mL min<sup>-1</sup>, and fractions of 2-mL were collected. All procedures were carried out at 4°C.

#### Mono Q (FPLC) Chromatography

Extracts from spinach leaf protoplasts were fractionated by FPLC, on a Mono Q HR 5/5 (Pharmacia) column at room temperature. After sample loading the column was washed with 5 mL equilibration buffer, and then eluted with the following gradient: 0 to 0.3 M NaCl (30 mL), 0.3 to 1.0 M NaCl (5 mL), 1.0 M NaCl (2 mL) in the same buffer, at a flow rate of 1.0 mL min<sup>-1</sup>, and 1-mL fractions were collected.

#### Enzyme Assays

Unless otherwise stated, enzymes were assayed spectrophotometrically at 340 nm, by coupling to reduction of NAD<sup>+</sup> or oxidation of NAD(P)H in 1 mL reaction volumes. All coupling enzymes supplied as suspensions in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were pelleted by centrifuging at 16,000g for 5 min and dissolved in 50 mM Hepes-NaOH (pH 7.6) before use. The reactions were started by addition of the last component given for each assay. The activity of each enzyme in a spinach leaf crude extract was shown to be dependent on the presence of all substrates and cofactors, and linear with respect to the amount of extract assayed.

#### ATP Sulfurylase (Sulfate Adenylyltransferase, EC 2.7.7.4)

Activity was measured as the pyrophosphate-dependent conversion of APS to ATP (9). The assay mixture contained: 50 mM Tris-HCl (pH 8.0), 5 mM MgCl<sub>2</sub>, 5 mM glucose, 0.3

mm NAD<sup>+</sup>, 0.1 mM APS, 5 units hexokinase (EC 2.7.1.1), 5 units glucose-6-phosphate dehydrogenase (EC 1.1.1.49, NAD<sup>+</sup> dependent from *Leuconostoc mesenteroides*) and 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>.

#### Glyceraldehyde-3-Phosphate Dehydrogenase (NADP<sup>+</sup>) (Phosphorylating), (EC 1.2.1.13)

Activity was measured as the conversion of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate (30). The assay mixture contained: 50 mM Hepes-NaOH (pH 8.2), 1 mM MgCl<sub>2</sub>, 4 mM EDTA, 0.2 mM NADPH, 5 mM DTT, 1 mM ATP, 10 units phosphoglycerate kinase (EC 2.7.2.3), 10 units triose-phosphate isomerase (EC 5.3.1.1), and 10 mM 3-phosphoglycerate.

#### Pyrophosphate:Fructose-6-Phosphate-1-Phosphotransferase (EC 2.7.1.90)

Activity was measured as the pyrophosphate-dependent conversion of fructose-6-phosphate to fructose-1,6-bisphosphate (30). The assay mixture contained: 50 mM Hepes-NaOH (pH 7.8), 0.5 mM MgCl<sub>2</sub>, 10  $\mu$ M fructose-2,6-bisphosphate, 5 mM fructose-6-phosphate, 10 units triose-phosphate isomerase (EC 5.3.1.1), 1 unit glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), 0.1 unit aldolase (EC 4.1.2.13), 0.15 mM NADH and 0.6 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>.

#### Hydroxypyruvate Reductase (EC 1.1.1.81)

Activity was measured according to Tolbert *et al.* (28). The assay mixture contained: 50 mM Mes-NaOH (pH 6.4), 0.2 mM NADH, and 1 mM hydroxypyruvate.

#### Citrate Synthase (EC 4.1.3.7)

Activity was measured as the oxaloacetate-dependent release of CoA from acetyl-CoA (10). The assay mixture contained: 50 mM Hepes-NaOH (pH 7.6), 0.25 mM MgCl<sub>2</sub>, 0.2 mM acetyl-CoA, 1.5 mM 5,5'-dithio-bis(nitrobenzoic acid) (DTNB), and 1 mM oxaloacetate. The reaction was followed by measuring the change in absorbance at 412 nm ( $\epsilon$  = 13,600 M<sup>-1</sup> cm<sup>-1</sup>).

#### O-Acetylserine(thiol)lyase (EC 4.2.99.8)

Activity was measured in a volume of 100  $\mu$ L containing: 50 mM K<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub> (pH 7.5), 5 mM DTT, 10 mM O-acetylserine, 2 mM Na<sub>2</sub>S. After incubation at 25°C for 10 min, the reaction was stopped by addition of 50  $\mu$ L 20% (w/v) TCA, and the precipitated protein was removed by centrifugation. Cysteine was measured in the supernatant by the method of Gaitonde (14). The supernatant was transferred to a glass tube containing 100  $\mu$ L concentrated acetic acid, and 200  $\mu$ L ninhydrin reagent (250 mg ninhydrin dissolved in 10 mL concentrated acetic acid:concentrated HCl 60:40 v/v). The tube was placed in a boiling water bath for 10 min, then cooled rapidly before addition of 550  $\mu$ L 95% (v/v) ethanol. Cysteine was determined by measuring the absorbance of the reaction mixture at 560 nm ( $\epsilon$  = 25,000 M<sup>-1</sup> cm<sup>-1</sup>).

### Serine Acetyltransferase (EC 2.3.1.30)

Activity was measured in extracts containing excess *O*-acetylserine(thiol)lyase activity by coupling the formation of *O*-acetylserine from serine and acetyl-CoA to the synthesis of cysteine. The reaction mixture contained: 4 mM serine, 2 mM acetyl-CoA, 50 mM  $K_2HPO_4$ - $KH_2PO_4$  (pH 7.5), 0.5 mM DTT, and 1 mM  $Na_2S$ . After incubation at 25°C for 30 min the reaction was stopped by addition of 50  $\mu$ L 20% TCA and cysteine determined as described above.

### Protein Determination

Protein was measured by the method of Bradford (5) using Bio-Rad protein assay reagent (Bio-Rad Laboratories GmbH, Munich, West Germany) with bovine  $\gamma$ -globulin as standard.

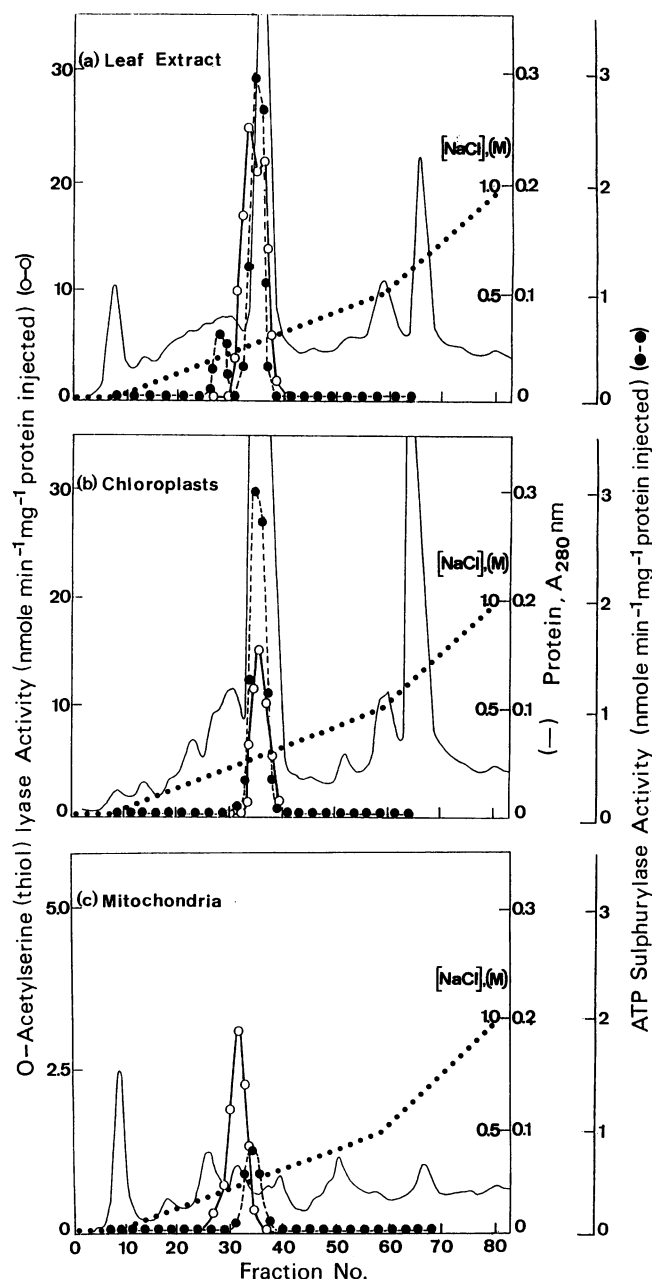
### Chl Determination

Chl was measured by the method of Arnon (2) modified according to Bruinsma (6).

## RESULTS

A crude extract from spinach leaves was fractionated by anion-exchange chromatography on a Q Sepharose column, and the fractions eluted from the column were assayed for ATP sulfurylase and *O*-acetylserine(thiol)lyase. ATP sulfurylase activity eluted in two distinct peaks (Fig. 1a), a minor peak eluting at a concentration of 210 mM NaCl and a major peak eluting at 280 mM NaCl, containing about 17 and 83% of the total activity, respectively. *O*-Acetylserine(thiol)lyase activity also eluted in two peaks (Fig. 1a), at 260 and 290 mM NaCl. The recoveries of ATP sulfurylase and *O*-acetylserine(thiol)lyase were 91 and 255%, respectively. Two peaks of ATP sulfurylase activity containing  $20 \pm 2\%$  and  $80 \pm 2\%$  (mean  $\pm$  SD of three experiments) were also found when crude extracts from spinach leaves were fractionated by anion-exchange chromatography on a Mono Q column.

To determine the intracellular origin of the different peaks in activity of ATP sulfurylase and *O*-acetylserine(thiol)lyase, extracts from isolated spinach leaf chloroplasts and mitochondria were fractionated on the Q Sepharose column. ATP sulfurylase activity from the chloroplasts eluted as a single peak at a concentration of 270 mM NaCl, and *O*-acetylserine(thiol)lyase also eluted as a single peak at 280 mM NaCl (Fig. 1b), with recoveries of 96 and 226%, respectively. The chloroplast extract was assayed for marker enzymes of chloroplasts (NADP-GAPDH), mitochondria (citrate synthase), peroxisomes (hydroxypyruvate reductase), and cytosol (PFP) and found to be essentially free of contamination by mitochondria, peroxisomes or cytosol (Table I). The ATP sulfurylase activity present in the mitochondrial extract eluted as a single peak at 270 mM NaCl, and the *O*-acetylserine(thiol)lyase activity eluted as a single peak at 240 mM NaCl (Fig. 1c), with recoveries of 85 and 52%, respectively. The mitochondrial extract was found to contain substantial activity of the peroxisomal marker enzyme, hydroxypyruvate reductase, and slight activity of the chloroplast marker enzyme, NADP-GAPDH, but was free of cytosolic contamination (Table I). The ratios of ATP sulfurylase to NADP-GAPDH



**Figure 1.** Separation of multiple forms of ATP sulfurylase and *O*-acetylserine(thiol)lyase from spinach leaves by chromatography on Q Sepharose. Spinach leaf extracts were fractionated by anion-exchange chromatography on a Q Sepharose Fast Flow column (50  $\times$  16 mm diameter) in 50 mM Tris-HCl, 5 mM  $MgCl_2$ , 2 mM DTT, 1 mM EDTA (pH 7.5) at 4°C. After sample loading, the column was washed with 10 mL buffer then eluted with a gradient of 0 to 0.5 M NaCl (100 mL), 0.5 to 1.0 M NaCl (50 mL) in the same buffer, at a flow rate of 0.2 mL min<sup>-1</sup>. Fractions of 2-mL were collected and assayed for ATP sulfurylase and *O*-acetylserine(thiol)lyase activity. The samples loaded were: (a), crude extract, 12.3 mg protein; (b), chloroplast extract, 28.6 mg protein; and (c), mitochondrial extract, 7.4 mg protein.

**Table I.** Distribution of ATP Sulfurylase, *O*-Acetylserine(thiol)lyase, and Marker Enzymes in Chloroplasts and Mitochondria from Spinach Leaves

ATP sulfurylase (ATP-S) and *O*-acetylserine(thiol)lyase (OAS[thiol]lyase) were assayed in a crude extract, isolated chloroplasts and mitochondria from spinach leaves along with marker enzymes to check for cross-contamination. The marker enzymes were: cytosol-PFP; chloroplasts-NADP-GAPDH; mitochondria-citrate synthase and peroxisomes-hydroxypyruvate reductase (HPR).

Sample	Enzyme Activity					
	PFP	NADP-GAPDH	Citrate synthase	HPR	ATP-S	OAS(thiol)lyase
	<i>nmol/min<sup>-1</sup> mg<sup>-1</sup> protein</i>					
Leaf extract	2.1	222	16	329	23.1	90.2
Chloroplasts	0.0	416	<1	18	18.5	58.5
Mitochondria	0.0	34	414	1,112	1.3	18.6

activity in the chloroplast and mitochondrial extracts were 0.044 and 0.038, respectively, whereas the ratios of *O*-acetylserine(thiol)lyase to NADP-GAPDH activity were 0.14 and 0.54, respectively. In two other preparations of spinach leaf mitochondria and one preparation of pea leaf mitochondria the activity of ATP sulfurylase was comparable to the degree of contamination by chloroplasts (data not shown). One preparation of spinach leaf mitochondria was further purified by a second gradient centrifugation. This procedure removed 95% of the peroxisomal contamination, but the activity of *O*-acetylserine(thiol)lyase was similar to that in nonpurified mitochondria (data not shown). The ratios of *O*-acetylserine(thiol)lyase to NADP-GAPDH activity in extracts of pea leaf chloroplasts and mitochondria were 0.15 and 0.42, respectively.

To determine whether the *O*-acetylserine(thiol)lyase found in the spinach leaf mitochondrial extract was truly within the

mitochondria, the latency of the enzyme was measured by comparing the activity in lysed and intact mitochondria. The mitochondria were lysed by suspension in hypotonic medium, followed by either three cycles of freeze-thawing or by addition of Triton X-100 (0.05%). The activities of *O*-acetylserine(thiol)lyase in freeze-thawed, Triton-treated and intact mitochondria were 20, 8, and 1.5 nmol min<sup>-1</sup> mg<sup>-1</sup> protein, respectively. After 15 min incubation, some cysteine synthesis occurred in intact mitochondria, probably due to gradual breakdown of the membranes.

To investigate the possibility that ATP sulfurylase and *O*-acetylserine(thiol)lyase are present in the cytosol, fractions enriched in chloroplasts, mitochondria or cytosol were prepared by differential centrifugation of lysed spinach leaf protoplasts. The distributions of ATP sulfurylase, *O*-acetylserine(thiol)lyase and marker enzymes in the three fractions are shown in Table II. The recoveries of each enzyme from fractionation of the protoplasts (Table II) were satisfactory, although the activity of *O*-acetylserine(thiol)lyase in the three fractions was significantly greater than the activity present in the original protoplasts. The chloroplast fraction contained substantial activity of the mitochondrial and peroxisomal marker enzymes, while the mitochondrial fraction was relatively free of chloroplasts although there was some peroxisomal contamination. Both the chloroplast and mitochondrial fractions were essentially free of cytosol. The cytosolic fraction contained some peroxisomal contamination but was relatively free of chloroplast and mitochondrial contamination. ATP sulfurylase activity was predominantly present in the chloroplast fraction with lower activities in the mitochondrial and cytosolic fractions. However, the percentage activity in the mitochondrial fraction was comparable to that of NADP-GAPDH in that fraction. *O*-acetylserine(thiol)lyase activity was also found in all three fractions. Although the activity was mainly in the chloroplast and cytosolic fractions, the

**Table II.** Distribution of ATP Sulfurylase and *O*-Acetylserine(thiol)lyase in Chloroplasts, Mitochondria, and Cytosol from Spinach Leaf Protoplasts

Spinach leaf protoplasts (0.2 mg Chl mL<sup>-1</sup>) were lysed by filtering twice through a 20 µm nylon mesh. Chloroplasts were pelleted by centrifuging 1-mL aliquots of the lysed protoplast suspension at 100g for 5 min. The supernatant was decanted and centrifuged at 5,000g for 5 min to give the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant). The chloroplast and mitochondrial pellets were each resuspended in 1 mL 50 mM Hepes-NaOH, 0.5 M sorbitol, 0.1% (w/v) BSA (pH 7.5). The fractions were assayed for ATP sulfurylase (ATP-S), *O*-acetylserine(thiol)lyase (OAS [thiol]lyase) and marker enzymes for cytosol-pyrophosphate: fructose-6-phosphate 1-phosphotransferase (PFP); chloroplasts-NADP<sup>+</sup> glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH); mitochondria-citrate synthase and peroxisomes-hydroxypyruvate reductase (HPR). Activities are expressed as percentages of the total activity recovered in the three fractions. The percentage recovery of each enzyme from the unfractionated protoplast lysate is also shown. Each value represents the mean ± SD from six measurements or four measurements for *O*-acetylserine(thiol)lyase.

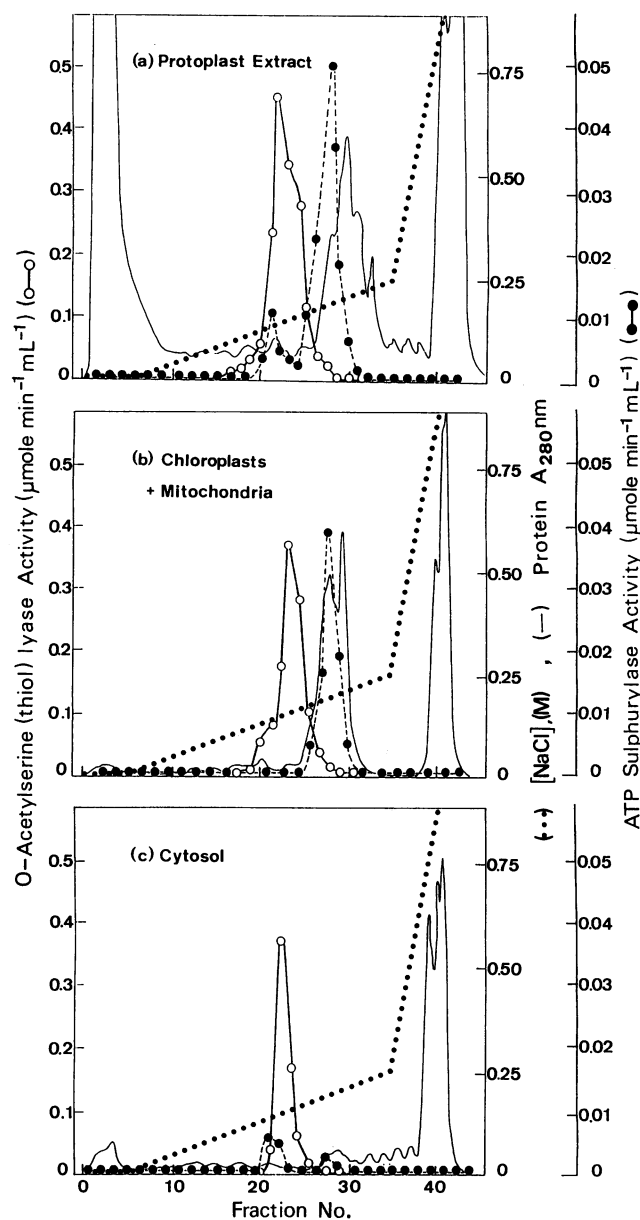
Sample	Enzyme in Fraction					
	PFP	NADP-GAPDH	Citrate synthase	HPR	ATP-S	OAS(thiol)lyase
	%					
Chloroplasts	3 ± 3	91 ± 2	39 ± 9	57 ± 5	76 ± 2	42 ± 2
Mitochondria	2 ± 2	6 ± 3	55 ± 9	23 ± 5	7 ± 2	14 ± 2
Cytosol	95 ± 3	3 ± 1	6 ± 2	20 ± 2	17 ± 1	44 ± 3
Recovery	110 ± 12	104 ± 5	89 ± 4	71 ± 5	104 ± 6	190 ± 10

percentage activity in the mitochondrial fraction was significantly greater than that of NADP-GAPDH.

To determine whether the activities of ATP sulfurylase and *O*-acetylserine(thiol)lyase found in the cytosolic fraction of the spinach leaf protoplasts represented distinct forms of each enzyme, extracts from protoplasts, combined chloroplasts + mitochondria and cytosol were fractionated on a Mono Q column. Both Mono Q and Q Sepharose have quaternary amines as their charged group and therefore display very similar chromatographic properties. The elution profile of ATP sulfurylase from the total protoplast extract on Mono Q (Fig. 2a) was very similar to that obtained previously for a crude extract on Q Sepharose (Fig. 1a), with a minor peak in activity eluting at 150 mM NaCl and a major peak eluting at 205 mM NaCl, containing about 16 and 84% of the total activity, respectively. ATP sulfurylase from the chloroplast + mitochondrial extract eluted as a single peak at 205 mM NaCl (Fig. 2b), whereas the enzyme from the cytosolic fraction eluted mainly in a peak at 150 mM NaCl, with a smaller peak at 210 mM NaCl (Fig. 2c) that was almost certainly due to contamination by the chloroplast enzyme. The recoveries of ATP sulfurylase activity from the protoplasts, chloroplasts + mitochondria and cytosol were 109, 89, and 59%, respectively. *O*-Acetylserine(thiol)lyase from the total protoplast extract eluted from the Mono Q column between 130 to 200 mM NaCl with a peak at 160 mM NaCl and distinct shoulder around 170 to 180 mM NaCl (Fig. 2a). The enzyme from the chloroplast + mitochondrial extract also eluted between 130 to 200 mM NaCl, but with a peak at 175 mM NaCl and a distinct shoulder around 130 to 140 mM NaCl (Fig. 2b), whereas *O*-acetylserine(thiol)lyase from the cytosolic fraction eluted as a single peak around 155 mM NaCl (Fig. 2c). The recoveries of *O*-acetylserine(thiol)lyase activity from the protoplasts, chloroplasts + mitochondria and cytosol were 169, 160, and 218%, respectively.

## DISCUSSION

Both ATP sulfurylase and *O*-acetylserine(thiol)lyase were found to be present as multiple forms in spinach leaves, with each form corresponding to a different intracellular compartment of the leaf. ATP sulfurylase was resolved into two forms with the more abundant form, comprising 83 to 84% of the total activity, located in the chloroplasts. The second form, comprising 16 to 17% of the total activity was absent from the chloroplasts, mitochondria and peroxisomes, but present in the cytosolic fraction of lysed spinach leaf protoplasts. It should be noted that the cytosolic fraction would also include the contents of the cell vacuole and endoplasmic reticulum, but it seems unlikely that the enzyme would be present in the vacuole. For brevity we shall refer to the enzyme as being cytosolic, although the possibility that the enzyme is present in the endoplasmic reticulum cannot be ruled out. We found no evidence for ATP sulfurylase activity in spinach leaf mitochondria. The low level of activity found in the isolated mitochondria was attributable to the presence of residual contamination by chloroplasts, because the ratio of ATP sulfurylase to NADP-GAPDH activity in the mitochondria was the same as that in the chloroplasts. In addition the ATP sulfurylase from the mitochondria behaved exactly like



**Figure 2.** Separation of multiple forms of ATP sulfurylase and *O*-acetylserine(thiol)lyase from spinach leaf protoplasts by chromatography on Mono Q. Spinach leaf protoplasts ( $0.2 \text{ mg Chl mL}^{-1}$ ) were lysed by filtering twice through a  $20 \text{ }\mu\text{m}$  nylon mesh, and  $3 \text{ mL}$  of the protoplast lysate were centrifuged at  $5,000g$  for  $5 \text{ min}$  to give the chloroplast + mitochondrial (pellet) and cytosolic (supernatant) fractions. The chloroplasts and mitochondria were lysed by suspension in  $3 \text{ mL}$   $10 \text{ mM}$  Hepes- $\text{NaOH}$ ,  $5 \text{ mM}$   $\text{MgCl}_2$  ( $\text{pH } 7.5$ ). A total protoplast extract was prepared by addition of Triton X-100, at a final concentration of  $0.2\%$  ( $\text{v/v}$ ), to  $3 \text{ mL}$  intact protoplasts. After centrifugation and desalting, the extracts were fractionated by FPLC on a Mono Q HR 5/5 column, in  $50 \text{ mM}$  Tris- $\text{HCl}$ ,  $5 \text{ mM}$   $\text{MgCl}_2$ ,  $2 \text{ mM}$  DTT,  $1 \text{ mM}$  EDTA ( $\text{pH } 7.5$ ), at room temperature. After sample loading the column was washed with  $2.5 \text{ mL}$  buffer then eluted with a gradient of  $0$  to  $0.3 \text{ M}$   $\text{NaCl}$  ( $30 \text{ mL}$ ),  $0.3$  to  $1.0 \text{ M}$   $\text{NaCl}$  ( $5 \text{ mL}$ ),  $1.0 \text{ M}$   $\text{NaCl}$  ( $2 \text{ mL}$ ) in the same buffer, at a flow rate of  $1.0 \text{ mL min}^{-1}$ . Fractions of  $1 \text{ mL}$  were collected and assayed for ATP sulfurylase and *O*-acetylserine(thiol)lyase activity. The samples loaded were: (a), total protoplast extract; (b), chloroplast + mitochondrial extract; and (c), cytosol.

the enzyme from chloroplasts during chromatography on Q Sepharose.

Chloroplasts from leaves of spinach, *Catharanthus*, maize (*Zea mays*), wheat (*Triticum aestivum*) and crabgrass (*Digitaria sanguinalis*) have been shown to contain ATP sulfurylase (25) and in oats (*Avena sativa*), maize, *Panicum miliaceum* and *Urochloa mosambicensis* the enzyme was found to be predominantly if not exclusively in the chloroplasts (9, 15). We found that in spinach leaves ATP sulfurylase is also located primarily in the chloroplasts, but our results clearly show that spinach leaves also possess a nonchloroplastic form of ATP sulfurylase, that is most probably located in the cytosol. Gerwick *et al.* (15) using electrophoresis also found two forms of ATP sulfurylase in leaves of *Panicum miliaceum*, but both forms were present in the bundle sheath cell chloroplasts. Paynter and Anderson (21) found multiple forms of ATP sulfurylase in hypocotyls of beetroot (*Beta vulgaris*) but only one form of the enzyme in leaves of the same species. These conflicting results may reflect differences between species; another possibility is that beetroot and *Panicum miliaceum* contain chloroplastic and cytosolic forms of ATP sulfurylase that were not resolved by the techniques used to study these two species.

The equilibrium constant of the ATP sulfurylase reaction lies heavily toward the substrates, ATP and  $\text{SO}_4^{2-}$ , therefore in order for the enzyme to work in the APS synthesizing direction, the other product of the reaction,  $\text{PPi}$ , must be removed. However, the cytosol of spinach leaves contains a concentration of 200 to 300  $\mu\text{M}$   $\text{PPi}$  (30). Therefore, it is not clear how the cytosolic ATP sulfurylase could maintain more than a minute concentration of APS in the cytosol, and so the role of the cytosolic ATP sulfurylase remains obscure.

*O*-Acetylserine(thiol)lyase activity from spinach leaves was resolved into three forms by anion-exchange chromatography. The enzyme was found mainly in the chloroplasts and in the cytosolic fraction from protoplasts. As noted before, the latter would also include the contents of the cell vacuole and endoplasmic reticulum, but it seems unlikely that the enzyme is in the vacuole. In contrast to the results for ATP sulfurylase the activity of *O*-acetylserine(thiol)lyase in the cytosolic fraction was as great as, if not greater than, the activity found in the chloroplasts. The enzyme was also found in both spinach leaf and pea leaf mitochondria. Although the mitochondria isolated directly from spinach leaves or from protoplasts were slightly contaminated by chloroplasts, this contamination could not account for all the *O*-acetylserine(thiol)lyase activity found in the mitochondria. In addition the mitochondrial enzyme did not behave like the chloroplast enzyme during chromatography on Q Sepharose, indicating that the two are distinct forms. The presence of *O*-acetylserine(thiol)lyase in spinach leaf peroxisomes cannot be ruled out, because the isolated spinach leaf mitochondria were substantially contaminated by peroxisomes. However, further purification of the mitochondria substantially reduced the peroxisomal contamination, but had little effect on the specific activity of *O*-acetylserine(thiol)lyase in the mitochondria; suggesting that the contaminating peroxisomes contributed little, if any, of the *O*-acetylserine(thiol)lyase activity seen in the isolated mitochondria. During chromatography of spinach leaf crude extracts and protoplast extracts, the peak of *O*-acetylser-

ine(thiol)lyase activity corresponding to the mitochondrial enzyme was masked by the more abundant cytosolic enzyme. However, the enzyme from cytosol-free mitochondria eluted from the Q Sepharose column at a lower salt concentration than the peak of activity in the crude extract corresponding to the cytosolic enzyme. The activity in mitochondria was shown to be latent, demonstrating that it was not due to binding of the enzyme from cytosol or broken chloroplasts to the mitochondrial envelope. The higher than expected recoveries of *O*-acetylserine(thiol)lyase activity were probably due to underestimation of the activity in the original extracts. This could arise if spinach leaves contain an inhibitor of *O*-acetylserine(thiol)lyase, that is separated from the enzyme or inactivated during anion-exchange chromatography or fractionation of protoplasts.

*O*-Acetylserine(thiol)lyase has been found in both the chloroplast and extrachloroplastic compartments of leaves, but the exact location of the extrachloroplastic enzyme was previously unknown (25). Our results now show that the extrachloroplastic *O*-acetylserine(thiol)lyase is located in both the cytosol and mitochondria of spinach leaves. We have also shown that the chloroplasts, cytosol and mitochondria possess distinct forms of *O*-acetylserine(thiol)lyase that can be resolved by anion-exchange chromatography. Multiple forms of *O*-acetylserine(thiol)lyase have been reported by several authors (4, 18, 20), but with the exception of Fankhauser and Brunold (13), who found that one form in spinach leaves was specific to the chloroplasts, no attempt has been made before to determine the intracellular origin of the different forms of *O*-acetylserine(thiol)lyase.

It is known that chloroplasts contain all the enzymes necessary for the assimilation of inorganic sulfate, and that isolated chloroplasts can synthesize [ $^{35}\text{S}$ ]cysteine from  $^{35}\text{SO}_4$  (25). Our results show that at least parts of the pathway are duplicated in the cytosol and mitochondria, but at present we do not know how far this duplication extends. Serine acetyltransferase has been found in spinach leaf chloroplasts (7) and mitochondria from *Phaseolus* leaves (27). Preliminary results from protoplast fractionation indicate that serine acetyltransferase is present in the chloroplasts, cytosol and mitochondria of spinach leaves. The synthesis of *O*-acetylserine requires a supply of acetyl-CoA. Both chloroplasts and mitochondria possess a pyruvate dehydrogenase complex that could supply acetyl-CoA using pyruvate from glycolysis (17) and in the cytosol ATP citrate lyase could supply acetyl-CoA using citrate exported from the mitochondria (16). It should also be noted that acetate is a by-product of the *O*-acetylserine(thiol)lyase reaction and that acetate can be converted to acetyl-CoA in the chloroplast by acetyl-CoA synthetase (17). A supply of serine must be available in the cytosol, chloroplasts and mitochondria for protein synthesis. Therefore, the serine acetyltransferase in each compartment can supply *O*-acetylserine for cysteine synthesis by the *O*-acetylserine(thiol)lyase also present in each compartment.

Although mitochondria from *Euglena* contain all the enzymes of the sulfate assimilation pathway (24), we know of no reports that the mitochondria or cytosol from leaves of higher plants contain the enzymes necessary for reduction of sulfite to sulfide, and in pea roots the enzymes are restricted to the plastids (8). If the chloroplasts alone have the ability to



reduce sulfite, this raises the question of where the sulfide needed for cysteine synthesis in the mitochondria and cytosol comes from. It is known that plants release  $H_2S$  when supplied with excess sulfate (22). If the excess sulfate is reduced in the chloroplasts, then sulfide must cross the chloroplast envelope in order to be released from the cell, suggesting that the chloroplast could supply sulfide for cysteine synthesis in the cytosol and mitochondria. Another possibility is that turnover of sulfur-containing proteins and other compounds in the cytosol and mitochondria could release sulfide that would be available for reassimilation by the mitochondrial and cytosolic *O*-acetylserine(thiol)lyases.

The chloroplasts are believed to be the sole site for the synthesis of lysine, threonine, phenylalanine, tyrosine, and tryptophan and the last enzyme in the pathway of methionine synthesis appears to be restricted to the cytosol (3), so it is perhaps surprising that there are three sites of cysteine synthesis in the cell. The presence of *O*-acetylserine(thiol)lyase in each of the three compartments where proteins are synthesized suggests that the plant cell may be unable to transport cysteine between compartments, so the cysteine required for protein synthesis must be synthesized *in situ*.

#### LITERATURE CITED

1. Anderson JW (1980) Assimilation of inorganic sulfate into cysteine. In BJ Miflin, ed, *The Biochemistry of Plants*, Vol 5. Academic Press, New York, pp 203–223
2. Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol* 24: 1–15
3. ap Rees T (1987) Compartmentation of plant metabolism. In DD Davies, ed, *The Biochemistry of Plants*, Vol 12. Academic Press, San Diego, pp 87–115
4. Bertognelli BL, Wedding R (1977) Purification and initial kinetic characterization of different forms of *O*-acetylserine sulphydrylase from seedlings of two species of *Phaseolus*. *Plant Physiol* 60: 115–121
5. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of dye-binding. *Anal Biochem* 72: 248–254
6. Bruinsma J (1961) A comment on the spectrophotometric determination of chlorophyll. *Biochim Biophys Acta* 52: 576–578
7. Brunold C, Suter M (1982) Intracellular localization of serine transacetylase in spinach leaves. *Planta* 155: 321–327
8. Brunold C, Suter M (1989) Localization of enzymes of assimilatory sulfate reduction in pea roots. *Planta* 179: 228–234
9. Burnell JN (1984) Sulfate assimilation in  $C_4$  plants: intercellular and intracellular location of ATP sulphydrylase, cysteine synthase and cystathionine  $\beta$ -lyase in maize leaves. *Plant Physiol* 75: 873–875
10. Cooper TG, Beevers H (1969) Mitochondria and glyoxysomes from castor bean endosperm—enzyme constituents and catalytic capacity. *J Biol Chem* 244: 3507–3513
11. Douce R, Bourguignon J, Brouquisse R, Neuburger M (1987) Isolation of plant mitochondria: general principles and criteria of integrity. *Methods Enzymol* 148: 403–415
12. Edwards GE, Gardestrom PE (1987) Isolation of mitochondria from leaves of  $C_3$ ,  $C_4$ , and Crassulacean acid metabolism plants. *Methods Enzymol* 148: 421–433
13. Fankhauser H, Brunold C (1979) Localization of *O*-acetylserine sulphydrylase in *Spinacia oleracea* L. *Plant Sci Lett* 14: 185–192
14. Gaitonde MK (1967) A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. *Biochem J* 104: 627–633
15. Gerwick BC, Ku SB, Black CC (1980) Initiation of sulfate activation: a variation in  $C_4$  photosynthesis plants. *Science* 209: 513–515
16. Kaethner TM, ap Rees T (1985) Intracellular location of ATP citrate lyase in leaves of *Pisum sativum* L. *Planta* 163: 290–294
17. Liedvogel B, Bauerle R (1986) Fatty acid synthesis in chloroplasts from mustard (*Sinapis alba* L.) cotyledons: formation of acetyl coenzyme A by intraplasmid glycolytic enzymes and a pyruvate dehydrogenase complex. *Planta* 169: 481–489
18. Masada M, Fukushima K, Tamura G (1975) Cysteine synthase from rape leaves. *J Biochem* 77: 1107–1115
19. Moore AL, Proudlove MO (1987) Purification of plant mitochondria on silica sol gradients. *Methods Enzymol* 148: 415–420
20. Nakamura K, Tamura G (1989) Five isoforms of cysteine synthase in rape leaves. *Agric Biol Chem* 53: 2537–2538
21. Paynter DI, Anderson JW (1974) Comparative enzymology of the adenosine triphosphate sulphydrylases from leaf and swollen hypocotyl tissue of *Beta vulgaris*—multiple enzyme forms in hypocotyl tissue. *Plant Physiol* 53: 180–186
22. Rennenberg H (1984) The fate of excess sulfur in higher plants. *Annu Rev Plant Physiol* 35: 121–153
23. Robinson SP (1987) Separation of chloroplasts and cytosol from protoplasts. *Methods Enzymol* 148: 188–194
24. Saidha T, Na S-Q, Li J, Schiff JA (1988) A sulphate metabolizing centre in *Euglena* mitochondria. *Biochem J* 253: 533–539
25. Schmidt A (1986) Regulation of sulfur metabolism in plants. *Progr Bot* 48: 133–150
26. Schwenn JD, Trebst A (1976) Photosynthetic sulfate reduction by chloroplasts. In J Barber, ed, *The Intact Chloroplast*. Elsevier-North Holland, Amsterdam, pp 315–334
27. Smith IK (1972) Studies of L-cysteine biosynthetic enzymes in *Phaseolus vulgaris* L. *Plant Physiol* 50: 477–479
28. Tolbert NE, Yamazaki RK, Oeser A (1970) Localization and properties of hydroxypyruvate and glyoxylate reductases. *J Biol Chem* 245: 5129–5136
29. Walker DA, Cerovic ZG, Robinson SP (1987) Isolation of intact chloroplasts: general principles and criteria of integrity. *Methods Enzymol* 148: 145–157
30. Weiner H, Stitt M, Heldt HW (1987) Subcellular compartmentation of pyrophosphate and alkaline pyrophosphatase in leaves. *Biochim Biophys Acta* 893: 13–21